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Decreased Azithromycin Susceptibility of *Neisseria gonorrhoeae* Due to *mtrR* Mutations

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Single-dose azithromycin therapy has recently been used in Uruguay for the treatment of uncomplicated gonococcal infections. As part of an active surveillance study to monitor the emergence of antibiotic resistance in gonococcal isolates, we examined the levels of azithromycin susceptibility in 51 consecutive isolates obtained from males with uncomplicated gonococcal urethritis. Isolates with decreased susceptibility to azithromycin (MICs, 0.25 to 0.5 μg/ml) were common, and these isolates often displayed cross-resistance to hydrophobic antimicrobial agents (erythromycin and Triton X-100). Resistance to erythromycin and Triton X-100 is frequently due to overexpression of the *mtrCDE*-encoded efflux pump mediated by mutations in the *mtrR* gene, which encodes a transcriptional repressor that modulates expression of the *mtrCDE* operon. Accordingly, we questioned whether clinical isolates that express decreased azithromycin susceptibility harbor *mtrR* mutations. Promoter mutations that would decrease the level of expression of *mtrR* as well as a missense mutation at codon 45 in the *mtrR*-coding region that would result in a radical amino acid replacement within the DNA-binding motif of MtrR were found in these strains. When these mutations were transferred into azithromycin-susceptible strain FA19 by transformation, the susceptibility of gonococci to azithromycin was decreased by nearly 10-fold. The *mtrCDE*-encoded efflux pump system was responsible for this property since insertional inactivation of the *mtrC* gene resulted in enhanced susceptibility of gonococci to azithromycin. We conclude that the *mtrCDE*-encoded efflux pump can recognize azithromycin and that the emergence of gonococcal strains with decreased susceptibility to azithromycin can, in part, be explained by *mtrR* mutations.

The emergence of strains of *Neisseria gonorrhoeae* that express clinically significant levels of resistance to penicillin and tetracycline is a global problem that is particularly acute in South America (9, 25). The loss of these relatively inexpensive antibiotics for the treatment of gonococcal infections has resulted in the use of alternative treatment regimens for effective clinical management of gonorrhea. For instance, azithromycin has been shown to achieve high and prolonged levels in tissues and cells, thereby allowing coverage against both gonococci and *Chlamydia trachomatis* (7). For these reasons, azithromycin has recently been used in Uruguay for the treatment of uncomplicated gonococcal infections in males.

Azithromycin is a 15-membered azalide derived from erythromycin by the replacement of the 9a carbonyl in the aglycone ring with a methyl-substituted nitrogen (35). Despite its improved pharmacokinetic properties and bactericidal capacity against gonococci in vitro, there is now evidence that clinical isolates with decreased azithromycin susceptibility can be recovered. Thus, we recently described (5) that for 161 consecutive genital isolates from males with acute gonococcal urethritis, the MIC at which 90% of isolates are inhibited (MIC_{90}) was 0.5 μg/ml. This result suggested that azithromycin resistance in gonococci was present in Uruguay and could pose significant problems for the effective management of patients with gonococcal infections.

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We have sought to determine the molecular basis for the decreased azithromycin susceptibility in strains of gonococci isolated from our patient population. In theory, resistance to azithromycin could arise due to mechanisms similar to those for resistance to other macrolides (10). These mechanisms include target site modification by methylases encoded by *erm* genes (18, 20, 21) or antibiotic inactivation due to the actions of different enzymes (1–4, 6, 17, 19, 22, 32) or the actions of efflux pumps (16, 36, 43). Little is known about the mechanisms of macrolide resistance in gonococci, but the recently described *mtrCDE*-encoded efflux pump has been suggested to be one mechanism by which certain strains could express decreased susceptibility to erythromycin (8, 12, 13, 23, 24, 33). This efflux pump has been shown to mediate energy-dependent export of structurally diverse hydrophobic antimicrobial agents. The *mtrCDE* genes constitute a single transcriptional unit that is negatively regulated by the product of the adjacent but divergent *mtrR* gene. The *mtrR* gene product (MtrR) is a transcriptional repressor that binds to a 31-nucleotide region that encompasses the promoter element that drives transcription of *mtrCDE* (23). Mutations within the *mtrR*-coding region or a 13-bp inverted repeat sequence within the *mtrR* promoter can result in decreased susceptibility of gonococci to erythromycin as well as other hydrophobic agents. We questioned whether such mutations might explain the decreased azithromycin susceptibility property that seems to be increasing in prevalence in isolates obtained from infected individuals in Uruguay. We now report that *mtrR* promoter and coding region mutations occur frequently in strains that express de-

TABLE 1. Susceptibilities of 51 clinical isolates of *N. gonorrhoeae* to tetracycline, erythromycin, and azithromycin

Antibiotic	MIC (μg/ml)		
	Range	50%	90%
Tetracycline[a]	0.25–8.0	2.0	8.0
Erythromycin	0.063–4	2.0	2.0
Azithromycin	0.032–0.5	0.25	0.5

[a] Isolates with plasmid-mediated resistance were excluded (MICs, ≥16 μg/ml).

creased susceptibility to azithromycin and that their presence can explain this property.

MATERIALS AND METHODS

Strains of *N. gonorrhoeae* used and growth conditions. A sample of 51 consecutive clinical isolates of *N. gonorrhoeae* with no known epidemiologic relationship were recovered from male patients with urethritis and were received in one of our laboratories (the laboratory of G.B. and L.Z.) for national (Uruguay) antimicrobial susceptibility surveillance studies. These strains were collected in Uruguay during 1996 and 1997. All isolates were identified as *N. gonorrhoeae* by conventional methods and were frozen at −70°C. Control strains (ATCC 49226, WHO III, WHO V, WHO VII, FA19, KH12, and KH15) with known profiles of susceptibility to the antimicrobial agents used in this study were included. Strain FA19 is considered a wild type with respect to the *mtrCDE*-encoded efflux pump system (13). Strains KH12 and KH15 are isogenic transformants of strain FA19. KH12 contains an insertional mutation in the *mtrC* gene (12) that renders it hypersusceptible to hydrophobic agents. Strain KH15 is hyperresistant to hydrophobic agents and contains a single-base-pair deletion in a 13-bp inverted repeat sequence within the promoter region used for transcription of the *mtrR* gene (13).

Antimicrobial testing. The MICs of azithromycin, crystal violet, erythromycin, Triton X-100, and tetracycline were determined by the agar dilution method as specified in the National Committee for Clinical Laboratory Standards protocol (29). Penicillinase production was tested with the chromogenic cephalosporin nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). High-level resistance to tetracycline was shown to be due to possession of *tetM* by PCR as described previously (26). By using the criteria of Morse et al. (28), strains that express high-level resistance to hydrophobic agents due to the *mtrCDE*-encoded system (Mtr phenotype) were identified with respect to their susceptibilities to crystal violet (MIC, >1.0 μg/ml), erythromycin (MIC, >2.0 μg/ml), and Triton X-100 (MIC, >2,000 μg/ml), as determined by the method of Shafer et al. (37).

PCR amplification and DNA sequencing studies. Chromosomal DNA was prepared from test strains as described previously (27). These DNA samples were used in PCRs to amplify the *mtrR* gene, including the promoter region, as described previously (23). The PCRs used the oligonucleotide primers used by Lucas et al. (23): RPMAL#2 (5′-ACTGAAGCTTATTTCCGGCGCAGGCAGGG-3′) and KH9#3 (5′-GACGACAGTGCCAATGCAACG-3′). The PCR products were purified by use of the QIAquick PCR purification kit protocol supplied by the manufacturer. Automatic DNA sequencing was performed at the Emory University DNA Sequencing Core Facility and used oligonucleotide primer RPMAL #2, KH9#1 (5′-GTCGCAGATACGTTGGAACAACG-3′), or KH9#3.

Transformation studies. In order to inactivate the *mtrCDE*-encoded efflux pump system in strains that express decreased susceptibility to azithromycin, four clinical isolates that express the Mtr phenotype were transformed with 0.5 μg of chromosomal DNA from strain KH12 (*mtrC*::Km^r) per ml. The transformation reaction was as described previously (12), and recombinants were selected for their resistance to kanamycin (50 μg/ml). Insertional inactivation of *mtrC* in transformants was confirmed by PCR with oligonucleotide primers KH9#2 (5′-CGTTTCGGGTCGGTTTGACG-3′) and KH9#3. The *mtrR* mutations in clinical isolate 9638 was introduced into strain FA19 by transformation with chromosomal DNA and selection for erythromycin-resistant transformants with 0.5 μg of erythromycin per ml. The susceptibilities of the transformants to antimicrobial agents along with that of the respective parental strain were determined as described above.

Statistical analyses. Linear correlations between logarithmic values of the MICs of azithromycin, erythromycin, and tetracycline were evaluated with the Pearson coefficient (r) with EPI INFO Statistical Software.

RESULTS AND DISCUSSION

Antibiotic susceptibilities of gonococcal isolates. We determined (Table 1) the susceptibilities of 51 consecutive isolates obtained from our patient population in Uruguay to azithromycin, erythromycin, and tetracycline; due to the high proportion (>50%) of β-lactamase-producing gonococci in Uruguay, penicillin susceptibility testing was not conducted. Chromosomally mediated resistance to tetracycline was observed in 62.7% (32 of 51) of the isolates, and seven additional isolates displayed higher levels of resistance to tetracycline (MIC, >16 μg/ml) due to a plasmid-borne *tetM* determinant. A high proportion of isolates displayed a multiple-drug-resistance pattern. There was a significant linear correlation between logarithmic values of the MICs of tetracycline or the MICs of azithromycin and logarithmic values of the MICs of erythromycin ($r = 0.77$ [$P < 0.01$] and $r = 0.84$ [$P < 0.01$], respectively) (data not shown).

Expression of Mtr phenotype in clinical isolates. Using the criteria of Morse et al. (28), we established that the Mtr phenotype (elevated resistance to crystal violet and Triton X-100) was expressed by nearly 50% of strains that displayed decreased susceptibility to azithromycin, erythromycin, and tetracycline (Table 2). It is relevant to note that those gonococci that displayed the Mtr phenotype also showed elevated resistance to azithromycin, erythromycin, and tetracycline compared to the level of resistance of those strains that did not display the Mtr phenotype.

Identification of *mtrR* mutations in clinical isolates. Previous studies (13, 33, 38) revealed that clinical isolates or labo-

TABLE 2. Susceptibilities of clinical isolates of *N. gonorrhoeae* with different cell envelope phenotype[a]

Antimicrobial agent and cell envelope phenotype	No. of isolates	No. of isolates for which MIC (μg/ml) was:									MIC (μg/ml)	
		0.032	0.063	0.125	0.25	0.5	1.0	2.0	4.0	8.0	50%	90%
Tetracycline[b]												
Mtr	21						1	4	8	8	4.0	8.0
Non-Mtr	23				1	1	8	9	3	1	2.0	4.0
Erythromycin												
Mtr	23							22	1		2.0	2.0
Non-Mtr	28		1	4	5	10	3	5			0.5	2.0
Azithromycin												
Mtr	23			2	9	12					0.5	0.5
Non-Mtr	28	2	4	7	13	2					0.25	0.25

[a] The Mtr cell envelope phenotype was defined for isolates for which the erythromycin MIC was ≥2 μg/ml, the Triton X-100 MIC was ≥2,000 μg/ml, and the crystal violet MIC was ≥1 μg/ml (28).

[b] Isolates with plasmid-mediated resistance were excluded (MICs, ≥16 μg/ml).

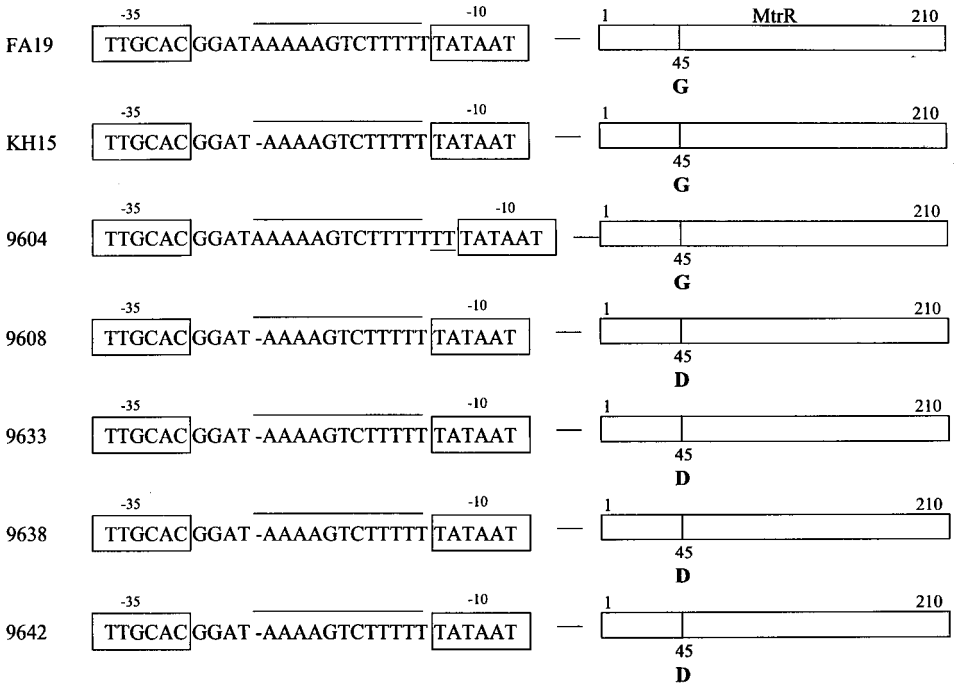

FIG. 1. *mtrR* mutations in clinical isolates of *N. gonorrhoeae*. Shown is the nucleotide sequence information of the *mtrR* promoter region from strains FA19 and KH15 and five clinical isolates that display decreased levels of azithromycin susceptibility. The −10 and −35 hexamers are shown inside the boxes, the presence of the 13-bp inverted repeat is shown with a line over the sequence, the single-base-pair deletion is shown with a dash, and the dinucleotide (TT) insertion in strain 9604 is underlined. Also presented is the Gly-45 (G)-to-Asp-45 (D) replacement in the helix-turn-helix motif of the MtrR proteins (210 amino acids in length) of strains 9608, 9633, 9638, and 9642.

ratory-derived mutants that display resistance to hydrophobic agents frequently contain loss-of-function mutations in the *mtrR*-coding sequence or a single-base-pair deletion in a 13-bp inverted repeat within the *mtrR* promoter. In order to determine whether the strains used in this study might contain mutations in the *mtrR* region, we randomly selected four strains (strains 9608, 9633, 9638, and 9642) that exhibited decreased susceptibility to azithromycin and erythromycin for molecular analysis. DNA was prepared from these isolates for PCR amplification of a nearly 1-kb sequence that encompassed the *mtrR*-coding and promoter sequences. DNA sequencing studies of these PCR products revealed (Fig. 1) that all four strains contained the previously described single-base-pair deletion in the *mtrR* promoter region. This mutation is known to abrogate transcription of *mtrR* while it enhances transcription of the *mtrCDE* efflux pump operon (14, 23). These effects result in high-level resistance to Triton X-100 (12, 38). In addition to this promoter mutation, each strain contained a missense mutation at codon 45, which results in a radical amino acid replacement (Gly-45 to Asp-45) within the helix-turn-helix motif of MtrR (Fig. 1); this mutation, in the absence of the promoter mutation, has been observed previously (13, 33, 38) in other gonococcal isolates that display intermediate levels of resistance to hydrophobic agents.

Transformation studies. In order to establish whether the *mtrCDE*-encoded efflux pump was responsible for the decreased azithromycin susceptibility expressed by strains 9608, 9633, 9638, and 9642, we inactivated their *mtrC* genes by transformation with the *mtrC*::Km^r determinant from strain KH12 (12). Insertional inactivation of *mtrC* in representative strains was confirmed by PCR analysis, as described in Materials and Methods (data not shown). In all cases, representative transformants of each clinical isolate displayed hypersusceptibility

TABLE 3. Results of MtrC inactivation in multiple-drug-resistant clinical isolates of *N. gonorrhoeae*

Isolate	MIC (μg/ml)		
	Triton X-100	Erythromycin	Azithromycin
9608	>16,000	2	0.25
9608 (*mtrC*::Km^r)[a]	16	0.032	0.032
9633	>16,000	2	0.25
9633 (*mtrC*::Km^r)[a]	16	0.064	0.032
9638	>16,000	2	0.25
9638 (*mtrC*::Km^r)[a]	16	0.064	0.032
9642	>16,000	2	0.5
9642 (*mtrC*::Km^r)[a]	16	0.064	0.064
9604	250	2	0.5
9604 (*mtrC*::Km^r)[a]	16	0.064	0.064
FA19 (wild type)	125	0.25	0.064
FA19 (*mtrR*-9638)[b]	>16,000	2	0.25
KH12 (*mtrC*::Km^r)[c]	16	0.032	0.032
KH15 (*mtrR-171*)[d]	>16,000	2	0.5

[a] Transformant of each clinical isolate obtained by transformation with DNA from strain KH12.

[b] Transformant of strain FA19 obtained by transformation with DNA from strain 9638.

[c] Strain KH12 is a transformant of FA19 in which the *mtrC* gene is inactivated by insertion of the 1.3-kb Km^r cassette, and it is hypersusceptible to a panel of hydrophobic agents (12).

[d] Strain KH15 contains the single-base-pair deletion in the 13-bp inverted repeat in the *mtrR-mtrC* promoter region (12, 14).

to azithromycin, erythromycin, and Triton X-100 (Table 3). This result confirmed that an intact *mtrCDE* operon was required for the hydrophobic agent resistance profiles expressed by these clinical isolates.

In order to verify that the mutations within *mtrR* are important in determining the decreased levels of azithromycin susceptibility, we transformed strain FA19 with chromosomal DNA from strain 9638. We selected erythromycin-resistant transformants and scored them for cross-resistance to azithromycin and Triton X-100. Representative transformants (see FA19 [*mtrR*-9838], Table 3) also had decreased susceptibility to azithromycin and Triton X-100. DNA sequencing analysis of the *mtrR* region from transformant strain FA19 (*mtrR*-9638) revealed that it had acquired both the single-base-pair deletion in the promoter and the missense mutation at codon 45 that are present in donor strain 9638 (data not shown). It is important that the single-base-pair deletion in the 13-bp inverted repeat sequence of the *mtrR* promoter was sufficient to decrease the level of susceptibility of gonococci to azithromycin by nearly 10-fold. This conclusion was drawn from a comparison of azithromycin MICs for isogenic strains FA19 and KH15 (Table 3), which differ at their *mtrR* regions by only the single base pair in the *mtrR* promoter (13).

Identification of a novel *mtrR* promoter mutation. The *mtrR* mutations in the clinical isolates described above were identical to those reported for other strains of gonococci that express resistance to multiple hydrophobic compounds (14, 39). During our screening of erythromycin-resistant clinical isolates, we noticed rare strains (e.g., strain 9604, Table 3) that, compared to strain FA19, displayed a nearly 10-fold decrease in susceptibility to azithromycin but only a 2-fold decrease in susceptibility to Triton X-100. We analyzed the importance of the *mtrCDE*-encoded efflux pump in determining levels of azithromycin and erythromycin susceptibility in strain 9604 by constructing an insertional mutation within its *mtrC* gene using donor DNA from strain KH12. A representative transformant of strain 9604 bearing *mtrC*::Km^r displayed significantly enhanced susceptibility to azithromycin, erythromycin, and Triton X-100 (Table 3). DNA sequence analysis of the *mtrR* region of strain 9604 revealed that it contained a heretofore undescribed mutation in its promoter. This mutation represented a dinucleotide insertion (TT) into the 13-bp inverted repeat sequence (Fig. 1). This mutation would increase the spacing between the −10 and −35 hexamers from an optimal 17 nucleotides to an unfavorable 19 nucleotides (14).

The combined genetic and molecular results obtained in this investigation implicate the *mtrCDE*-encoded efflux pump as a mechanism by which gonococci can express decreased susceptibility to azithromycin. Through mutations that are known (13) to abrogate transcription of the gene (*mtrR*) that encodes a transcriptional repressor of *mtrCDE* or loss-of-function mutations in the repressor-encoding gene, gonococci can overproduce the MtrC-MtrD-MtrE efflux pump to increase their capacity to export hydrophobic agents. These hydrophobic agents are structurally diverse and include dyes (e.g., crystal violet), detergents (Triton X-100), and drugs (erythromycin and azithromycin). That azithromycin can be recognized by this efflux pump system is not surprising given its structural similarity to erythromycin. Other bacterial efflux pumps (30, 31, 34), including the MexA-MexB-OprM pump of *Pseudomonas aeruginosa*, which displays similarity at the amino acid level to the MtrC-MtrD-MtrE pump of gonococci (8, 13, 14, 40), can also recognize macrolide antibiotics.

Over the past decade, several studies have evaluated the efficacy of a single, oral dose of azithromycin for treatment of bacterial sexually transmitted diseases, including gonorrhea. Although a single, oral dose of 1.0 g of azithromycin proved effective for treatment of uncomplicated chlamydial infections, treatment of uncomplicated gonococcal infections produced variable results (15, 41, 45). In this respect, failure of azithromycin therapy for patients with uncomplicated gonococcal urethritis has been reported (11, 41, 42, 44, 46). The azithromycin MICs for some gonococcal strains isolated from patients who have failed azithromycin therapy were, in fact, similar to the MICs obtained for the isolates used in this investigation (0.25 to 0.5 μg/ml). Our results demonstrate that *mtrR* mutations that are known to result (12, 14, 23) in overproduction of the MtrC-MtrD-MtrE efflux pump are sufficient to result in these unfavorable azithromycin MICs. The acquisition of additional mutations outside of the *mtr* gene complex that modify the azithromycin structure or its target could further enhance azithromycin resistance to levels that would abolish the effectiveness of azithromycin as an alternative antibiotic in the treatment of uncomplicated gonorrhea. Continued surveillance and monitoring of antibiotic susceptibility patterns are needed to detect the emergence of such strains.

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